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ORIGINAL ARTICLE

Differential Prox-1 and CD 31 expression in mucousae, cutaneous and soft tissue vascular lesions and tumors

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Abstract

The study of lymphatic vessels and lymphatic tumors has been hampered with difficulty due to the overlapping morphological features between blood and lymphatic endothelial cells, as well as to the lack of specific lymphatic endothelial markers. Over the last few years, lymphatic vessels and lymphangiogenesis have received great attention owing to their putative implications in terms of metastatic dissemination and the promise of targets for lymphangiogenic therapy. Prox-1 is a nuclear transcription factor that plays a major role during embryonic lymphangiogenesis and is deemed to be a useful marker for differentiating lymphatic endothelial cells from the other blood vessels endothelial cells. Here, we describe a double-immunostaining strategy for formalin-fixed, paraffin-embedded tissues that aims at evaluating the distribution of Prox-1 and CD 31 – a cytoplasmic pan-endothelial marker – in a series of 28 mucousae, cutaneous and soft tissue vascular lesions and tumors, including hemangiomas, lymphangiomas, lymphangiectasia, and Kaposi's sarcomas. Our results showed that in non-lesional mucousae and skin, Prox-1 decorated exclusively the nuclei of endothelial cells in lymphatic vessels. Prox-1 stained almost all the benign lymphatic vascular lesions/tumors (91%) and was absent or only focally positive in 75% of blood vascular tumors. CD 31 stained endothelial cells of blood vessels of superficial and deep dermal plexuses, lymphatics, and all blood vascular lesions/tumors. Kaposi's sarcomas were all positive for both CD 31 and Prox-1 markers. In conclusion, although Prox-1 expression in vascular lesions/tumors was not entirely restricted to tumors with known lymphatic differentiation, CD 31/Prox-1 double-immunolabeling can be used as an adjunct marker to identify lymphatic vessels in routinely processed formalin-fixed, paraffin-embedded samples.

Keywords: Prox-1; CD 31; Kaposi's Sarcoma; Hemangioma; Lymphangioma

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Introduction

The lymphatic network is a complex system composed of variably sized vascular channels lined by specialized endothelial cells, which drain interstitial fluid and lymph from peripheral tissues and return them to venous system for recirculation [13,14,18,19]. The study of lymphatic vessels and lymphatic tumors was long forgotten due to the overlapping morphological features between blood and lymphatic endothelial cells, as well as to the lack of specific lymphatic endothelial markers [13,14,18,19].

Recently, several new lymphatic markers have been developed such as VEGFR-3 [4,6,13,14,18,19], LYVE-1 [6,9,13,14,18,19], podoplanin [4,13,14,18,19], D2-40 [10], and Prox-1 [2,6,13–19,27,28]. Regardless of their sensitivity for lymphatic endothelial cells, VEGFR-3, podoplanin, and LYVE-1 fall short in specificity for these cells [6,9,13,14,16,18,19,30]. VEGFR-3 is usually restricted to lymphatic vessels in normal adult tissues, but is re-expressed by blood endothelial cells in wound healing and in tumor-induced neovessels [13,14,19,30]. LYVE-1 was originally reported to be specific for lymphatic endothelial cells of cutaneous and solid organs [9,13,14,18,19]; however, it is consistently expressed in endothelial cells of the hepatic sinusoids [6,14]. Moreover, very recent studies demonstrated that in experimental models of xenotransplanted tumors, LYVE-1 also decorates capillaries in the alveolar walls, as well as occasional lung arteries and veins [16]. Several lines of evidence indicate podoplanin-specific expression in lymphatic endothelial cells [4,13,14], but in vitro models showed that only a fraction of podoplanin-positive endothelial cells also co-express LYVE-1 [12]. D2-40 is a highly sensitive lymphatic marker and seems to be useful for distinguishing vascular from lymphatic vessels [10]. However, this marker cross-reacts with leiomyosarcoma cells, malignant fibrous histiocytoma cells, and dermatofibroma cells [10].

Prox-1, a homeobox gene cloned by homology with the Drosophila gene *prospero*, is a nuclear transcription factor that plays a major role during embryonic lymphangiogenesis and in the differentiation of various neuroectodermal structures [2,15,17,23,27,28]. Prox-1 knockout mice lack a terminally differentiated lymphatic system. Since Wigle and Oliver's seminal study [27], Prox-1 has been considered a specific and sensitive lymphatic endothelial cell marker. At the mRNA level, the only non-lymphatic endothelial cells expressing Prox1 are found on the concave side of the cardiac valves [23]. Interestingly, Prox-1 is the first described nuclear marker for lymphatic endothelial cells [6,14,27,28].

We herein describe the distribution of Prox-1 in normal skin and in a series of vascular lesions/tumors by double-immunostaining with CD 31, a cytoplasmic pan-endothelial marker [24], and Prox-1, a nuclear lymphatic endothelial cell marker.

Material and methods

Patients and tumor samples

Formalin-fixed, paraffin-embedded tissue blocks of vascular lesions were retrieved from the pathology files of the Hospital S. João, Porto, Portugal, and from the Institute of Molecular Pathology and Immunology of University of Porto (IPATIMUP), Porto, Portugal. All cases were independently reviewed by two pathologists (JML and JSRF) and classified according to the new World Health Organisation classification [7,26]. Discordant cases were resolved on a multi-head microscope. Only those cases in which an agreement was achieved were included in the study. In 28 cases, both pathologists agreed upon the diagnoses, including 9 capillary hemangiomas, 3 cavernous hemangiomas, 1 spindle cell hemangioma, 6 lymphangiomas, 3 cystic lymphangiomas, 1 lymphangioma *circumscriptum,* 1 lymphangiectasia, and 4 cutaneous Kaposi's sarcomas. Formalin-fixed, paraffin-embedded tissue blocks of all cases were available. Table 1 summarizes the clinicopathologic features of the cases.

Immunostaining procedures

The double-immunostaining technique was carried out using a primary antibody raised against Prox-1 [2, 6] and a commercial antibody against CD 31 (Neomarkers, clone JC/70A, Freemont, CA, USA). Briefly, sections were immersed in antigen retrieval solution (DAKO, Glostrup, Denmark) and microwaved at 600 w for 20 min. Sections were further incubated with Ultravision block solution (Neomarkers, Freemont, CA, USA) for 10 min at room temperature before incubation of 60 min at room temperature with the first primary antibody (Prox-1, 1:1000). Sections were sequentially washed in PBS and incubated with biotinylated goat anti-polyvalent immunoglobulin for 10 min, streptavidin-peroxidase for 10 min, and developed with 3,3′-diaminobenzidine for 10 min. Sections were also sequentially incubated with goat serum for 20 min, then with the second primary antibody (CD 31, 1:25) for 60 min at room temperature, labeled with goat anti-mouse immunoglobulin for 30 min, and then with APAAP (*alkaline phosphatase–antialkaline phosphatase* complex) for 30 min. Slides were developed with fast-red dye (Fast Red Tablet, LabVision Co., Freemont, CA, USA) for 35–40 min and mounted in aquatex medium (Merck, Dermstadt, Germany).

Table 1. Summary of the clinical pathologic features of the lesions

Case	Gender	Age	Site	Histopathological diagnosis
1	F	10 months	Arm	Capillary hemangioma
2	M	9	Face	Capillary hemangioma
3	F	19	Finger	Capillary hemangioma
4	M	2	Hand	Capillary hemangioma
5	M	63	Oral cavity	Capillary hemangioma
6	F	15	Shoulder	Capillary hemangioma
7	F	63	Skin	Capillary hemangioma
8	M	1	Thorax	Capillary hemangioma
9	M	11	Face	Capillary hemangioma
10	F	5	Forearm	Cavernous hemangioma
11	F	33	Knee	Cavernous hemangioma
12	F	44	Neck	Cavernous hemangioma
13	M	41	Gum	Spindle cell hemangioma
14	M	51	Face	Lymphangioma
15	F	10 months	Mediastinum	Lymphangioma
16	F	4	Neck	Lymphangioma
17	M	7	Thigh	Lymphangioma
18	F	21	Vulva	Lymphangioma
19	M	2	Tongue	Lymphangioma
20	M	2	Axilla	Cystic lymphangioma
21	M	5	Axilla	Cystic lymphangioma
22	M	Newborn	Neck	Cystic lymphangioma
23	F	53	Vulva	Lymphangioma circumscriptum
24	F	47	Oral cavity	Lymphangectasia
25	M	22	Face	Kaposi's sarcoma
26	F	68	Thigh	Kaposi's sarcoma
27	F	42	Multifocal	Kaposi's sarcoma
28	M	48	Multifocal	Kaposi's sarcoma

Immunohistochemistry evaluation

For the immunohistochemical analysis, the expression of CD 31 and Prox-1 was independently evaluated by two pathologists (JML and JSRF) in a blinded fashion and not being aware of the histopathological diagnosis. A semiquantitative analysis for the distribution of both markers in endothelial cells was performed according to the following scoring system: negative (−): absence of expression; focal positive (±): expression in up to 10% of the vascular structures; positive (+): expression in more than 10% of vascular structures.

Positive reactions were assessed in hot spot areas in which proliferating vascular structures were present and stained. Negative controls of reactions were performed by substituting the immune sera with non-immune rabbit serum or negative control rabbit IgG or by omitting the primary antibodies. As Prox-1 is consistently expressed in retinal neuroepithelial cells [25], sections of normal human retina were used as positive controls, whereas for CD 31, sections of a lymphangioma of vulvae were used. All controls were satisfactorily stained in each slide run.

Results

In the skin/mucousae adjacent to the vascular lesions/tumors, Prox-1 stained only the nuclei of endothelial cells in vascular structures with very thin walls comprising only endothelial cells, without red blood cells in their luminal spaces, consistent with lymphatic vessels [22]. Double staining disclosed a clear differential pattern when comparing blood-type with lymphatic-type vessels: both types of vessels showed endothelial cell cytoplasm stained with fast-red (CD 31), whereas only lymphatic type vessels disclosed endothelial cell nuclei stained with brown DAB (Prox-1) (Fig. 1A). Blood vessels with morphologically evident pericytic or smooth muscle layers, epidermal, squamous epithelial, appendageal, and dermal or corion structures were consistently negative for Prox-1. CD 31 strongly decorated endothelial cells of blood vessels of the superficial and the deep dermal plexus. In intra-ocular tissues, used as a control, PROX-1 decorates the nuclei of the inner nuclear layer of the retina (Fig. 1B) whereas none of the endothelial cells in the intra-ocular vessels stained for Prox-1 (Fig. 1B). This was expected, as

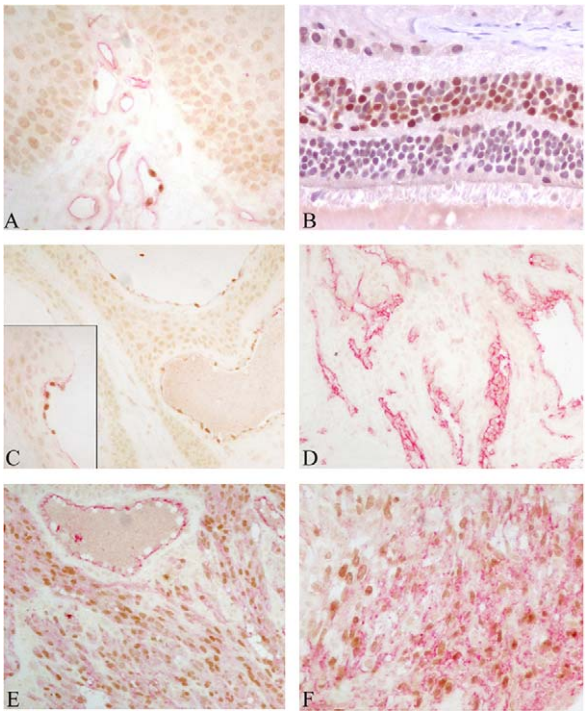

Fig. 1. Appearance of Prox-1 and CD 31 immunohistochemistry in normal controls and vascular lesions. (A) Prox-1 positivity (brown) in endothelial nuclei of lymphatics and negativity in blood vessels of oral mucosae. Both types of vessel show endothelial cytoplasmic (red) CD 31 positivity (Double Prox-1/CD 31 immunostaining). (B) Prox-1 expression (brown) in the nuclei of the inner nuclear layer of the retina. Of note is the absence of Prox-1 expression in endothelial cells (Single Prox-1 immunostaining, counterstained with hematoxylin). (C) Lymphangioma showing endothelial cells with Prox-1 nuclear (brown) and CD 31 cytoplasmic (red, inset) positivity (Double Prox-1/CD 31 immunostaining). (D) Hemangioma showing CD 31 endothelial cytoplasmic positivity (red) and absence of nuclear Prox-1 reactivity (Double Prox-1/CD 31 immunostaining). (E) Spindle cell hemangioma nuclear Prox-1 (brown) and cytoplasmic CD 31 (red) co-expression in tumor cells of the spindle cell component. The hemangiomatous component shows CD 31 endothelial cytoplasmic positivity (red) and absence of nuclear Prox-1 reactivity (Double Prox-1/CD 31 immunostaining). (F) Kaposi's sarcoma showing nuclear Prox-1 (brown) and cytoplasmic CD 31 (red) co-expression in tumor cells (Double Prox-1/CD 31 immunostaining).

lymphatic channels are absent in the intra-ocular compartment.

Prox-1was observed in all but one lymphatic-derived tumors 10/11 (91%) (Fig. 1C, Table 2). Prox-1 was negative in 7/12 (58%) blood vessel tumors (Fig. 1D, Table 2). The positive blood vessels tumors were 3 capillary hemangiomas and 2 cavernous hemangiomas (Table 2). The spindle cell hemangioma (Fig. 1E) showed a peculiar distribution of CD 31/Prox-1: spindle cells were consistently positive for both markers,

Table 2. Expression of Prox-1 and CD 31 in vascular lesions and tumors

Case	Histopathological diagnosis	CD 31	Prox-1
1	Capillary hemangioma	+	–
2	Capillary hemangioma	+	+
3	Capillary hemangioma	+	–
4	Capillary hemangioma	+	+
5	Capillary hemangioma	+	–
6	Capillary hemangioma	+	–
7	Capillary hemangioma	+	–
8	Capillary hemangioma	+	–
9	Capillary hemangioma	+	±
10	Cavernous hemangioma	+	+
11	Cavernous hemangioma	+	–
12	Cavernous hemangioma	+	±
13	Spindle cell hemangioma	+	+[a]
14	Lymphangioma	+	–
15	Lymphangioma	+	+
16	Lymphangioma	+	+
17	Lymphangioma	+	+
18	Lymphangioma	+	+
19	Lymphangioma	+	+
20	Cystic lymphangioma	+	+
21	Cystic lymphangioma	+	+
22	Cystic lymphangioma	±	+
23	Lymphangioma circumscriptum	+	+
24	Lymphangectasia	±	±
25	Kaposi's sarcoma	+	+
26	Kaposi's sarcoma	+	+
27	Kaposi's sarcoma	+	+
28	Kaposi's sarcoma	+	+

–, Absence of expression; ±, up to 10% of positive cells; +, more than 10% of positive cells.

[a]Positivity was restricted to the spindle cell component. Dilated blood vessels were lined by Prox-1-negative endothelial cells.

whereas endothelial cells lining dilated vessels were negative for Prox-1, but positive for CD 31, thus reflecting a complex admixture of different cell types in these tumors. On the other hand, in lesions with known lymphatic histogenesis/differentiation, CD 31 showed moderate to strong staining in most of the cases, except in 2 lymphatic-derived lesions (cases 22 and 24), which showed less than 10% of positive cells (Table 2). Kaposi's sarcomas were strongly positive for both CD 31 and Prox-1 markers (Fig. 1F, Table 2).

Discussion

Prox-1 has now been established as an essential fate-determining factor for lymphatic endothelial cell expression in the developing vessels. Rediscovering the lymphatic system has had a significant impact on cancer therapy, namely prompting the development of specific

drugs that may block the spread of cancer cells from the primary site through lymphatic conduits. For this particular purpose, the identification of specific lymphatic markers is of utmost importance [1,11,14,21,22].

Differentiating blood from lymphatic vessels is particularly difficult in certain circumstances even for experienced pathologists. Morphological characteristics of endothelial cells, particularly under neoplastic conditions, are not clear-cut and do not allow for a proper distinction between blood and lymphatic vessels. This becomes even more complicated in vascular tumors and vascular malformations, which happen to be lesions of complex histogenesis comprising both blood and lymphatic vessels [29].

As Prox-1 is also expressed in some non-endothelial cells such as hepatocytes, bile ductum epithelium, pancreatic epithelium, central nervous system, lens, retina, and cardiomyocytes in avian and murine embryos [22,29], a CD 31/Prox-1 double-immunolabeling method proved cautious and very useful to identify lymphatic vessels, in particular in reactive and neoplastic lesions, where blood and lymphatic vessels frequently harbor morphological and protein regulation different from that of their non-neoplastic counterparts [8,20,29].

The importance of vascular studies in neoplasias has been intensively studied [5]. Angiogenesis and lymphangiogenesis are crucial for the understanding of tumor biology and for designing potential cancer preventive and therapeutic measures, since both vasculature systems probably cross talk with each other and share common signaling factors [1].

We present some data concerning the distribution of Prox-1 in vascular lesions and tumors. Twenty out of 28 cases were positive for Prox-1 (71.4%). Most importantly, 9 out of 10 lesions with known lymphatic differentiation were positive for this marker (sensitivity = 90%). Wilting et al. [29] have studied the expression of Prox-1 and CD 31, among other markers, to differentiate blood and lymphatic vessels using immunofluorescent markers in a series composed by fetal intestine, skin lymphedema, hemangioma, and lymphangioma. Particularly in hemangiomas, they found the co-expression of CD 31 and CD 34 (a marker frequently expressed in blood vessels), but not Prox-1. Conversely, they found Prox-1, CD 31, and VEGFR-3 expression, but not CD 34 in lymphangiomas. In our series, Prox-1-positive endothelial cells were observed in 3 capillary and 2 cavernous hemangiomas. Two conclusions may be drawn from these findings: (i) gene expression patterns of vascular tumors are more complex than previously appreciated and (ii) although remarkably sensitive, Prox-1 is not entirely restricted to lymphatic neoplastic vessels. Therefore, caution should be exercised when using Prox-1 as a lymphatic marker in this context. In fact, in our view, Prox-1 should rather be used as a nuclear lymphatic marker in conjunction with other cytoplasmic antibodies for identifying lymphatic differentiation in vascular neoplasms. However, Prox-1 can be reliably used for the identification of lymphatic vessels in non-vascular tumor and reactive/inflammatory samples.

The histogenesis/differentiation displayed by neoplastic endothelial cells of Kaposi's sarcomas is still a contentious issue [3,8]. In the present study, Kaposi's sarcomas were consistently positive for both CD 31 and Prox-1. Our results are in agreement with those of previous reports on the lymphatic nature of Kaposi's sarcomas using other lymphatic markers, including VEGFR-3, LYVE-1, and D2-40 [3,8,10,31]. Taken together, these results demonstrate that Kaposi's sarcomas should be perceived as tumors with lymphatic vessel differentiation.

The spindle cell hemangioma showed a characteristic distribution, exhibiting positivity for Prox-1 in the spindle cell areas and no staining for this marker in the nuclei of endothelial cells lining dilated blood vessels. As the distribution of Prox-1/CD 31 staining in the spindle cell component of the spindle cell hemangioma and Kaposi's sarcomas is remarkably similar, our double-immunostaining method cannot be used for differentiating these two lesions. On the other hand, the similar reactivity pattern does suggest that the spindle cell component of both, Kaposi's sarcoma and spindle cell hemangioma, may harbor features of early lymphatic differentiation.

In summary, our results demonstrate that Prox-1 staining is a feasible tool to identify lymphatic vessels in routinely processed formalin-fixed, paraffin-embedded samples. In addition, CD 31/Prox-1 double-immunolabeling proved useful owing to their high sensitivity of these antibodies as markers of blood and lymphatic endothelial cells, respectively. Prox-1 expression in vascular lesions/tumors was not entirely restricted to tumors with known lymphatic differentiation. This fact opens interesting fields for further research on the upregulation of Prox-1 in neoplastic scenario. Finally, our results support the suggestion of the lymphatic nature of Kaposi's sarcomas [3].

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